# LECTURE-27

## Microarrays for PTM Analysis

## Handout

### PREAMBLE

Proteins undergo chemical modifications at certain amino acid residues following translation. These modifications are essential for normal functioning of protein and are carried out by one or more enzyme catalyzed reactions. PTMs are vital cellular control mechanism, known as “cellular switches” that affect protein properties such as protein folding, conformation, activity and functions. As a result they play very important role in various diseases. Phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis are some of the important PTMs. PTMs increase the functional diversity of proteome and influence almost all aspects of cellular activities. Most common example of PTMs is phosphorylation and dephosphorylation, which regulates various important cellular events such as signaling cascades, cell cycle, etc. Therefore, comprehensive analysis of PTMs is essential to understand signaling and metabolic pathways. Protein and antibody microarray provides useful platform for PTM analysis in a high-throughput manner. In this lecture we will discuss the applications of protein microarrays for studying post-translational modifications.

**OUTLINE OF LECTURE**

I. Significance of post-translational modification

II. Methods for studying post-translational modifications

III. Applications of microarray in studying PTMs

IV. Challenges

V. Conclusions

**Box-1. Terminology**

**Protein microarrays:** Miniaturized arrays normally made of glass, onto which small quantities of many proteins can be simultaneously immobilized and analyzed. For detection of phosphorylation sites, potential protein substrates are immobilized on to the array.

**Kinase:** An enzyme that is responsible for phosphorylation of specific amino acid residues in the protein with the help of ATP as a phosphate donor.

**Phosphorylated proteins:** Proteins that have been phosphorylated at specific amino acid residues (Serine, Threonine or Tyrosine).

**Antibody microarrays:** An array onto which different antibodies are spotted, which has specific binding domains for detection of protein of interest from a complex mixture. For detection of PTMs, antibodies against specific protein motifs containing the PTM or against a specific residue containing a phosphorylated site may be used.

## I. SIGNIFICANCE OF POST-TRANSLATIONAL MODIFICATIONS

Proteome is typically of higher complexity than genome. Although, the human genome comprised of ~ 25,000 genes, the total number of proteins in the human proteome is comparatively very large. This indicates that more than one protein can be expressed by a single gene (IHGSC 2004; Jensen et al 2004). Different mRNA transcripts are generated through genomic recombination, using different transcription initiation and/or termination site, and by alternative splicing from a single gene (Ayoubi et al. 1996). Another level of complexity in the human proteome is generated by PTMs. PTMs are chemical modifications, which occur at side chains of amino acids or at peptide linkage of a protein by enzymatic activity, and these modifications play a key role in functional proteomics as they regulate cellular activities, localization and interaction of proteins with other cellular molecules. It is estimated that 5% of the genes in the human genome encode enzymes that perform more than 300 types of post-translational modifications (Walsh et al. 2006).

The chemical modifications that take place at certain amino acid residues after the protein is synthesized by translation are known as post-translational modifications (PTMs). These are essential for normal functioning of the protein. There are several types of post-translational modifications that can take place at different amino acid residues. The most commonly observed PTMs include phosphorylation, glycosylation, methylation as well as hydroxylation and acylation. Many of these modifications, particularly phosphorylation, serve as regulatory mechanisms for protein action.

PTM generate tremendous diversity and are extremely important. Many documented effects of PTMs include – change in enzymatic activity, ability to interact with other proteins, sub-cellular localization and targeted degradation. Phosphorylation of amino acid residues is carried out by a class of enzymes known as kinases that most commonly modify side chains of amino acids containing a hydroxyl group. Phosphorylation requires presence of a phosphate donor molecule such as ATP, GTP or other phoshorylated substrates. Removal of phosphate groups are carried out by phosphatase enzyme and it forms an important mechanism for regulation of proteins. Glycosylation involves linking saccharides to proteins in presence of glycosyltransferases enzymes, giving rise to a glycoprotein. Glycosylation plays vital role in various biological functions such as antigenicty of immunological molecules, cell division, protein targeting stability and interactions. Aberrant glycosylation forms result into various human congenital disorders. Some of the most important PTMs are listed in Table 1.

**Table 1:** Type of post-translational modification and significance

| **Type of post-translational** | **Amino acid residue involved** | **Significance** |
|---|---|---|
| Phosphorylation and dephosphorylation | Serine, Threonine, and Tyrosine | Regulate cellular processes including cell cycle, growth, apoptosis and signal |
| Glycosylation | Aspargine, serine, Threonine | Influence protein folding, conformation, distribution, stability and activity |
| Ubiquitination | Lysine | Mediate degradation of protein |

| | | |
|---|---|---|
| Methylation | Nitrogen or oxygen to amino acid side | Epigenetic regulation<br>N-methylation is irreversible while O- |
| S-Nitrosylation | Cysteine | Stabilize proteins, regulate gene expression and provide NO donors |
| N-Acetylation | N-terminal methionine | Biological significance of N-acetylation is not clear |
| | μ-$NH_2$ of lysine | Regulation of Gene expression |
| N-myristoylation | N-terminal glycine | Target proteins to plasma membranes |
| S-palmitoylation | Thiolate side chain of cysteine | Target proteins to plasma membranes |
| S-prenylation | Cysteine residues within 5 amino | Proteolytic cleavage by Rce1 and methylation by isoprenyl cysteine methyl |

## II. METHODS FOR STUDYING POST-TRANSLATIONAL MODIFICATIONS

Studying post-translational modifications is particularly important as PTMs plays pivotal role in cellular physiology and gets altered in diseases. Few of the PTMs have been extensively investigated at the proteome level. The challenges in studying PTMs are the development of specific detection and purification methods. With continuous advancement in technologies, it is possible to meet these challenges. Various methods can be employed for studying the PTMs, such as mass spectrometry (MS) based proteomics technologies for global PTM analysis, Western blotting and protein microarrays. Antibody microarray provides a useful platform for PTM analysis in a high-throughput manner. There are various types of microarrays specialized for the identification of specific PTMs (Figs 1-3; Table. 2).

**Table 2**. Microarray platform for PTM analysis

| **Type of Microarray** | **Applications** |
|---|---|
| Peptide array | Characterization of kinase specificity |
| Antibody arrays | Network analysis of phosphorylated proteins |
| Oligosaccharide | Protein–carbohydrates interactions |
| Lectin arrays | Characterizing glycoproteins |

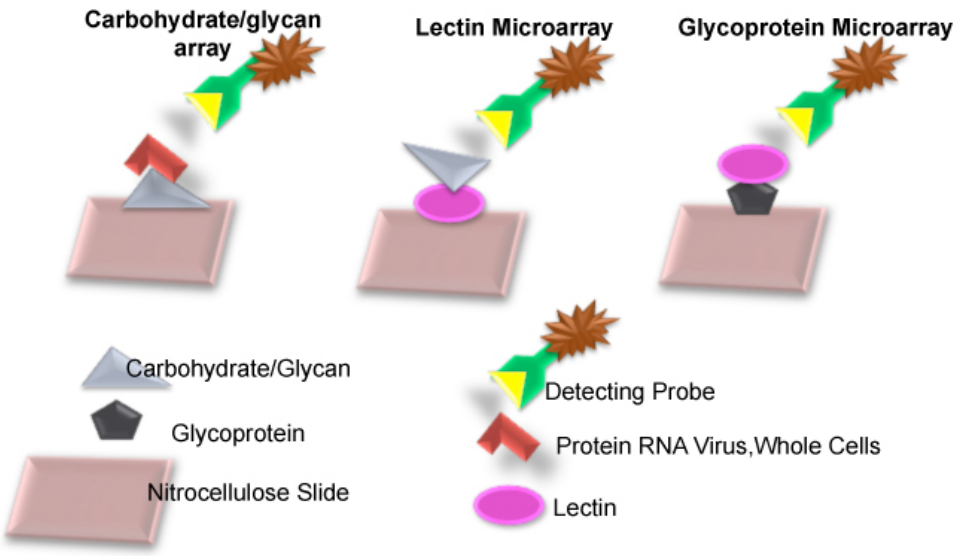


**Fig 1.** For glycosylation studies different types of microarray platforms such as carbohydrate array, lectin arrays, glycoprotein arrays and other array formats have been used.

**Protein microarrays**

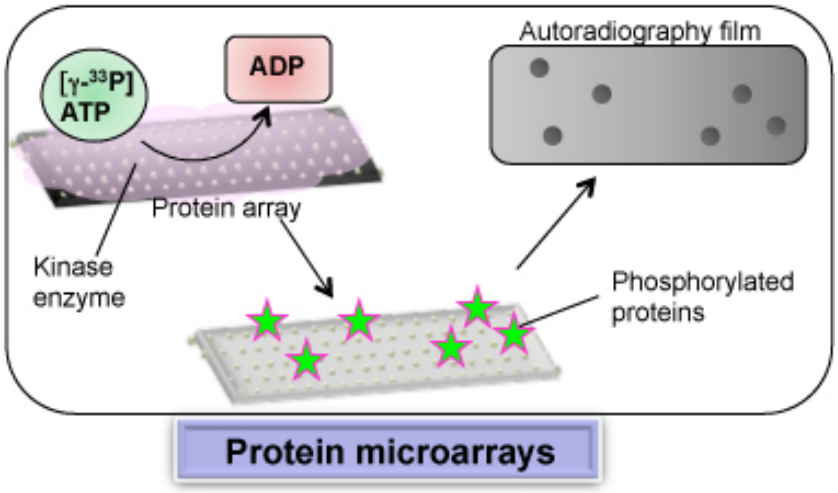


**Fig 2.** Protein microarrays for PTM analysis

***Illustration: Microarray-based detection techniques for PTMs – Protein Microarrays***

*PTMs such as phosphorylation can be detected by means of protein microarrays using a kinase assay. Potential substrates for protein phosphorylation are immobilized on a suitably coated array surface. To this, kinase enzyme and gamma P-32 labeled ATP are then added and the array is incubated at 30°C. The phosphorylation reaction occurs at those sites containing proteins that can be modified. After sufficient incubation, excess unbound ATP and enzyme are washed off the array surface. Detection is carried out by means of autoradiography wherein a photographic film is placed in contact with the array surface. The radioactive emissions from the phosphate label present at the phosphorylated protein sites strike the film. Upon development, the positions at which phosphorylation has occurred can be clearly determined. Thus proteome chip technology offers a useful platform for detection of phosphporylated proteins.*

**Antibody Microarrays**

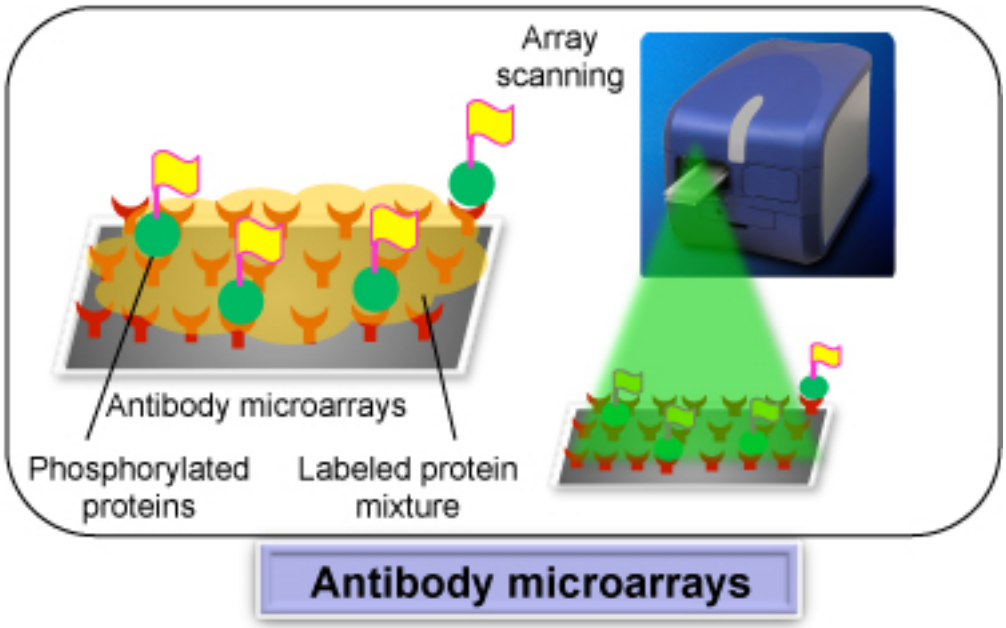


**Fig 3.** Antibody microarrays for PTM analysis

***Illustration: Microarray-based detection techniques for PTMs – Antibody Microarrays***

*Antibodies specific to phosphorylated serine, threonine or tyrosine residues as well as motif antibodies can be immobilized on to a suitably coated microarray surface and used for detection of PTM. The complex protein mixture containing modified and unmodified proteins is labeled with a suitable fluorescent tag molecule and added to the array surface. Specific binding interactions occur between the phosphorylated proteins and their corresponding antibodies. The array is then washed to remove any excess unbound proteins from the surface. This is followed by scanning of the array using a microarray scanner at a suitable wavelength to detect the fluorescent tag of the bound proteins. This method offers sensitive and simultaneous detection of large number of post-translationally modified proteins.*

## III. APPLICATIONS OF MICROARRAYS IN STUDYING POST-TRANSLATIONAL MODIFICATIONS

### Global analysis of protein phosphorylation in Yeast

Snyder and colleagues performed a global analysis of protein phosphorylation, using whole proteome arrays for the study of protein kinases in *Saccharomyces cerevisiae* (Ptacek, J. et al., 2005). There were 5800 out of 6200 open reading frames (ORFs) from yeast that were cloned into expression vector carrying two tags; an N-terminus gluthathione S-transferase (GST) and oligohistindine tags. Proteins were expressed and purified by affinity chromatography. Proteins were spotted in duplicate onto nickel-coated slides and purified kinases were used to modify them. Phosphorylation substrates on yeast proteome chips were identified by incubating slides with individually 87 yeast protein kinases in the presence of γ-33P ATP. To identify proteins those undergo auto-phosphorylation, proteome chips were incubated with γ-33P ATP in kinase buffer and phosphorylation was detected using auto-radiography (Figs 4-5).

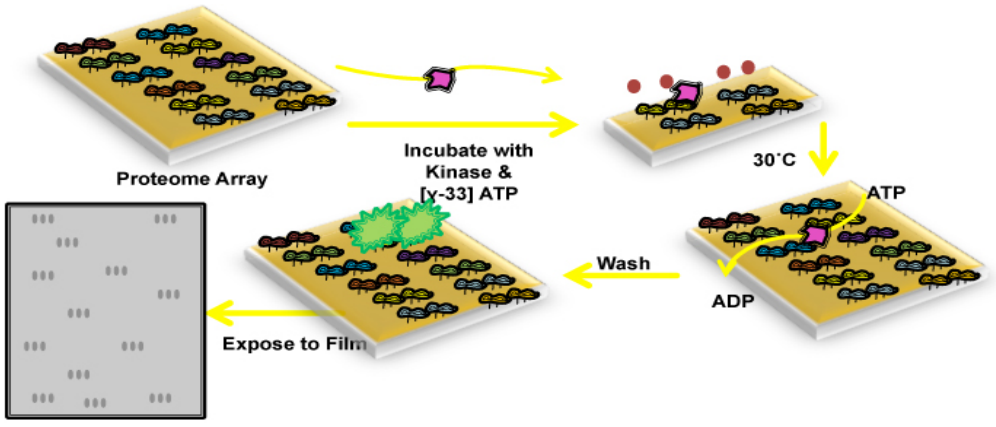


**Fig 4.** Schematic of PTM analysis to identify kinase substrates: yeast proteome array was prepared by spotting about 4,400 proteins in duplicates and each kinase was over-

expressed, purified and assayed on yeast proteome chips and detection was done autoradiographically.

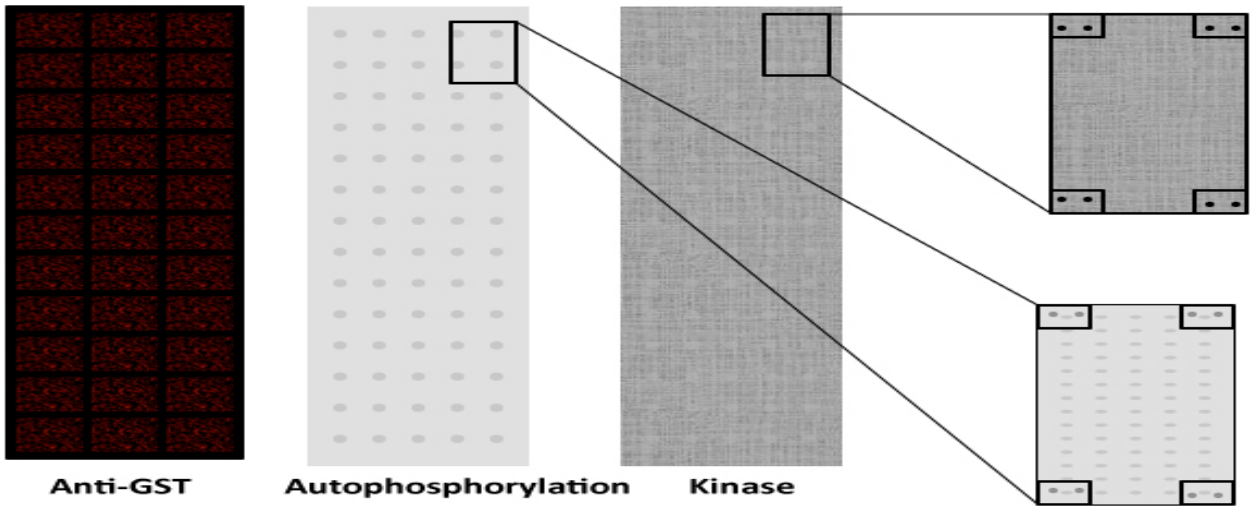


**Fig 5.** Kinase assays on protein chips; slide on the left side represents the quality of proteome chip as all fusion proteins has GST tag; middle slide shows the autophosphorylation signals and four corners of the two boxes of the slides on the right side show signal for auto-phosphorylation from kinases printed on slides.

This study created a map of yeast phosphoproteome and identified several phosphorylation events. Protein kinases showed specificity for substrates and 73% of substrates were recognized by specific few kinases, indicating a strong preference of kinases for specific substrates.

**Detection of lysine methylation on proteins using microarrays**

Lysine methylation is one of the key post-translational modifications, which plays an important role in several biological activities, including epigenetic regulation. Levy et al. (2011) used the ProtoArray platform, which contained more than 9500 human proteins and identified new substrates for protein lysine methyltransferase (PKMT) enzymes, SETD6 and SETD7. SETD6 methylates Rel A on lys 310 residue, and SETD7 is a histone methyltransferase, which methylates histone H3 at lys4 position. Fluorescent (Pan-methyl antibody) and radioactive assays (radioactively labeled SAM) were used for detection. Fluorescent-based detection identified 321 positive candidates for SETD7, while 118 positive candidates for SETD6 and results were consistent with radioactivity based method. 26 candidates were found to be common for SETD6 in both the detection methods. Further, *in vitro* validation was performed for six candidates (TCEA1, PAK4, RPS27L, SFRS2, PLK1, and DNAJC8) and *in vivo* validation for PLK1 and PAK4 in 293T cells by immuno-precipitation and western blotting.

**Identification of protein kinase substrates using protein microarray**

Protein arrays have been successfully used for identification of substrate for kinases. In one such study, Meng et al (2008) identified 23 substrates and further validated CAMK2, while 4 candidates (FLJ22795, SH3YL1, CRKL, and ABI1) were identified as substrate for ABL kinase (Fig 6). It was demonstrated that time, buffer compositions, and protein concentrations affect the assay; therefore assay parameters should be optimized before carrying out functional assay.

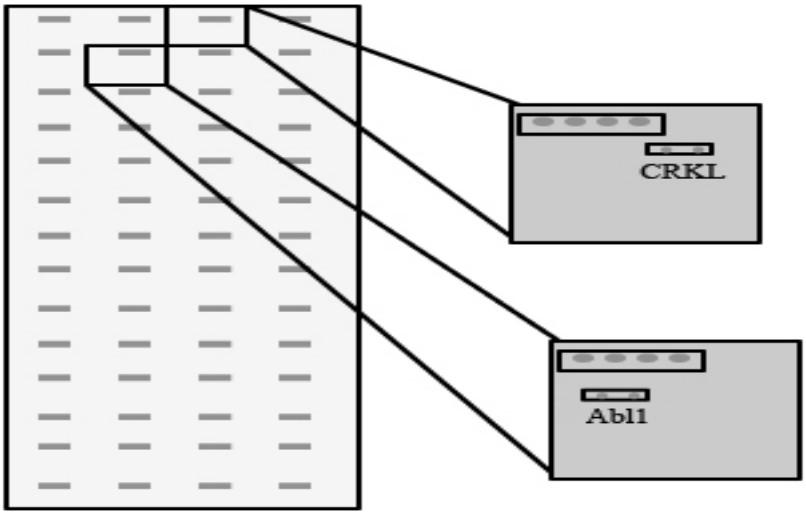


**Fig 6.** Identification of Abl kinase substrate using protein microarrays

In 2009, Yifat Merbl and Marc W. Kirschner (2009) have successfully used microarray approach to study substrates for ubiquitination using cell extracts prepared from cells released from mitotic checkpoint.

## IV. CHALLENGES

PTMs regulate catalytic activities of several proteins thus influence their biological functions. The protein microarray platforms have shown promising results for PTM study; however, these approaches have certain limitations:

- Limited number of proteins due to difficulty in protein expression and purification
- Tethering of proteins on surface may results in loss of their functionality
- Unavailability of specific antibodies
- Sensitivity and dynamic range of detection systems
- Complications in studying PTMs (other than phosphorylation) such as ubiquitination, methylation etc which play important role in biological activities
- Microarray platform cannot portray *in vivo* conditions hence findings should be validated by other confirmatory experiments

## V. CONCLUSIONS

PTMs help in proper protein folding or stability of several proteins, where these modifications occur shortly after translation is completed. PTMs can be reversible depending on nature of the modification. For example, phosphorylation of a protein at specific amino acid position can activate the catalytic or biological function of a protein; conversely, phosphatases hydrolyze the phosphate group to remove it from the protein and reverse the biological activity or vice-versa. Several proteomic techniques have been employed for studying PTMs. The protein microarrays have overcome many limitations associated with other proteomics techniques, and shown its applications in studying several types of PTMs in high-through manner. However, unavailability of specific antibodies, sensitivity and dynamic range of detection systems etc. are some of the challenges associated with use of protein array approach, which need to be resolved. Studying PTMs still remains challenging. Many advanced proteomic technologies have attempted to bridge this gap; however, no single technique can be solely relied for screening all the PTMs in a given biological question. Further advancements in protein microarray technology will help to study PTMs in comprehensive manner.

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